Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi: 10.1016/S1995-7645(14)60266-2

Cytotoxic activity of the methanolic extract of leaves and rhizomes of *Curcuma amada* Roxb against breast cancer cell lines

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ARTICLE INFO

Article history:
Received 29 Apr 2014
Received in revised form 18 May 2014
Accepted 15 Jun 2014
Available online 2 Aug 2014

Keywords
Curcuma amada
Cancer
Viability
Apoptosis
Cell lines
Cytotoxicity

ABSTRACT

Objectives: To evaluate the methanol extract of both the leaves and the rhizomes of *Curcuma amada* (*C. amada*) for their cytotoxic activity against breast cancer cell lines MCF-7 and MDA MB 231.

Methods: Viability and cytotoxicity induced by the extracts were assessed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, sulforhodamine B, and lactate dehydrogenase release assays. Various staining techniques such as acridine orange/ ethidium bromide, Giemsa, ethidium bromide, propidium iodide, and Hoechst 33342 staining were employed to study the mechanism of cell death induced by the extract.

Results: The results indicated that the methanol extract of both the leaves and the rhizomes of *C. amada* exhibited strong cytotoxicity towards breast cancer cell lines MCF-7 and MDA MB 231. The extract also showed less cytotoxicity towards non-cancerous breast cell line HBL-100. The results of staining revealed that the extracts induced cell death in cancer cells which are mediated through apoptotic pathway.

Conclusions: The results indicated that the methanol extract of leaves and rhizomes of *C. amada* possess anticancer and cytotoxic activity.

1. Introduction

Cancer is one of the most dreadful diseases that is globally distributed among the world's population. Cancer occurs due to excessive free radical damage which ultimately causes damage to the genetic material DNA, protein and lipids. This DNA damage leads to mutations which cause normal cells to transform into a cancer cell[1]. Failure of apoptosis and increased rate of cell survival occurs due to DNA damage that results in cancer development. Apoptosis is the major form of programmed cell death which takes place in all the cells to maintain homeostasis and cellular integrity. Cancer treatment therapies target this apoptotic pathway by increasing apoptosis in cells and thus preventing cancer[2,3].

Breast cancer is the most common type of cancer among women in both developed as well as developing countries[4]. The incidence of breast cancer cases has been increasing among Indian women and it has been estimated that by the end of 2030, there will be about 200 000 new breast cancer cases[5,6]. In India about 1 out of 28 women has breast cancer during her life time. This incidence is 1 out of 22 in rural areas and in urban areas 1 out of 60 women are prone to the development of breast cancer.

There are three most common types of breast cancers which are classified based on the receptors expressed in these cells. They are estrogen/ progesterone positive (ER+/PR+), HER 2 positive (HER^{2+}) and triple negative breast cancer (ER^-, PR^-, HER^{2-}). The natural products derived from the medicinal plants provide an excellent source of cancer medication. The use of natural products in cancer therapy offers many advantages because in addition to killing the

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cancer cells, some natural agents have been found to render protection to normal cells[7].

Curcuma amada (*C. amada*) Roxb is a well known rhizomatous herb which is commonly known as mango ginger that belongs to the family Zingiberaceae (ginger family) that is widely cultivated in various parts of South India. *C. amada* rhizomes have been used for culinary purposes and pickle preparations in South India. Our previous study have shown that the methanol extract possess good antioxidant activity and also did not induce cytotoxicity towards yeast cells. Hence, the present study was formulated to evaluate the cytotoxic activity of the methanol extract of leaves and rhizomes against estrogen receptor positive and triple negative breast cancer cell lines MCF-7 and MDA MB 231 respectively. A non-cancerous breast cell line HBL-100 was also used for comparison.

2. Materials and methods

2.1. Plant material

C. amada Roxb rhizomes were procured from Arya Vaidya Pharmacy, Centre for Indian Medicinal Plant Heritage, Kanjikode, Kerala and were grown as pot culture in our university herbal garden and were identified by Botanical Survey of India, Southern Circle. Both fresh leaves and rhizomes were collected for the study. Previous studies conducted by us showed that the methanol extract of the leaves and rhizomes were rich with antioxidants.

2.2. Extract preparation

The fresh leaves and rhizomes collected were rinsed with tap water, blotted dry using a filter paper and used for extract preparation. The components present in the leaves and rhizomes were extracted using methanol. The methanol extract prepared after evaporation of methanol was dissolved in dimethylsulfoxide (0.2 mg/mL-IC_{50} dose).

2.3. Culturing of cell lines

All the cell lines MCF-7, MDA MB 231 and HBL-100 were purchased from National Centre for Cell Science, Pune, India. The cell count was done and the cell viability was tested by trypan blue using haemocytometer. The cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% non-essential amino acids in tissue culture flasks and incubated in a CO_2 incubator in a 5% CO_2 and 95% humidity atmosphere. Once the cells attained confluent growth, the cells were trypsinized using Trypsin-EDTA (PAA) and the required number of cells 10^6 and 10^3 cells/mL were seeded into 6-well and 96-well plates respectively for carrying out various assays. Cell viability and cytotoxicity assays were carried out in 96-well plates and the staining was performed in 6-well plates. In each well of the 6-well plates, a clean, dry, sterile cover slip was placed before the cells were seeded, followed by incubation in a CO_2 incubator in a 5% CO_2 and 95% humidity atmosphere (Innova CO-170, United States).

2.4. Treatment groups

MCF-7, MDA MB 231 and HBL-100 were treated with the leaf and rhizome extract (0.2 mg/mL) for 24, 18 and 12 h respectively which was the optimal treatment time of the extracts in each of the cell lines. The effect induced was also compared to the standard drugs used *viz.* tamoxifen for estrogen receptor positive MCF-7 and HBL-100 cells and etoposide for triple negative MDA MB 231 cells. The following treatment groups are set up of the study. Negative control: cells alone. Positive control: cells+ tamoxifen/ etoposide. Test groups: cells+ methanol extract of *C. amada* leaves; cells+ methanol extract of *C. amada* leaves+ tamoxifen/ etoposide; cells+ methanol extract of *C. amada* rhizomes; cells+ methanol extract of *C. amada* rhizomes+ tamoxifen/ etoposide.

2.5. Viabiltiy assays

2.5.1. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay

The treated cells (100 μL), were incubated with 50 μL of MTT at 37 °C for 3 h. After incubation, 200 μL of phosphate-buffered saline (PBS) was added to all the samples. The liquid was then carefully aspirated. Then 200 μL of acid-propanol was added and left overnight in the dark. The absorbance was read at 650 nm in a microtitre plate reader (Anthos 2020, Austria). The optical density of the control cells were fixed to be 100% viability and the percent viability of the cells in the other treatment groups were calculated[8].

2.5.2. Sulforhodamine B (SRB) assay

A portion of 350 μL of ice-cold 40% trichloroacetic acid was layered on top of the treated cells and incubated at 4 °C for one hour. The cells were then washed 5 times with 200 μL of cold PBS. The buffer was removed; about 350 μL of SRB stain was added to each well and left in contact with the cells for 30 min at room temperature. The unbound dye was removed by washing 4 times with 350 μL portions of

1% acetic acid. Then 10 mmol/L tris (350 μL) was added to each, to solubilize the protein-bound dye and the plate was shaken gently for 20 min. The tris layer in each well was transferred to a 96-well plate and the absorbance was read in a microtitre plate reader (Anthos 2020, Austria) at 492 nm. The cell survival was calculated as the percent absorbance compared to the control (untreated) cells[9].

2.5.3. Lactate dehydrogenase (LDH) release assay

The cells were treated with the leaf and rhizome extracts in the presence and absence of the oxidant, H_2O_2, for 1 h incubation at 37 °C. Similarly, a spontaneous control (PBS alone) and maximum LDH release control (cells in PBS lysed using lysis buffer) were also taken. After incubation, the cells were centrifuged and 50 μL of the supernatant was taken into a new ELISA plate reader; A total of 50 μL of the reconstituted substrate mix (dissolved one vial of the provided substrate mix in 11.4 mL water and added 0.6 mL of assay buffer provided with the kit) was added to each well and incubated at room temperature for 30 min. The reaction was stopped by adding 50 μL of stop solution and the absorbance was recorded at 490 nm and the percent cytotoxicity was calculated as follows:

$$\%\ \text{Cytotoxicity}=\frac{\text{Experimental}-\text{Spontaneous}}{\text{Maximum LDH release}}\times 100$$

2.6. Observation of morphological and nuclear changes

2.6.1. Acridine orange/ethidium bromide (AO/EtBr) staining

To the treated cells in cover slips, 10 μL of AO/EtBr was added and spread by placing a cover slip over it. The stained slides were incubated at room temperature for five minutes. The apoptotic cells were visualized by their green fluorescence which was counted by using an upright fluorescent microscope using B2A filter at 400× magnification[10].

2.6.2. Giemsa staining

The diluted Giemsa stain (10 μL) was added to the slide and spread by placing another cover slip over it. The cells were then observed for morphological changes using a phase contrast microscope (Nikon, Japan) at 400× magnification[11].

2.6.3. Ethidium bromide staining

Ethidium bromide (10 μL) was added to the treated cells and spread by placing a coverslip over it. The slides were incubated for 5 min at room temperature. The apoptotic cells with condensed chromatin and fragmented nuclei were counted by using fluorescent microscope (Nikon, Japan) using G-2A filter at 400× magnification[12].

2.6.4. Propidium iodide staining

The treated cells were washed with PBS to remove traces of medium and serum. The cells were permeabilized with 50 μL of acetone: methanol (1:1) mixture at −20 °C for 10 min. Then 10 μL of propidium iodide (PI) was added, spread by placing a cover slip over it and incubated at 37 °C for 30 min in the dark[13].

2.6.5. Hoechst 33342 staining

The treated cells were harvested and washed with PBS twice, and then incubated with Hoechst 33342 (1 μg/mL) for 30 min at 37 °C. The stained cells were visualized under an inverted fluorescence microscope (Moticam, Hong Kong) using Hoechst filter at 400× magnification[14].

2.7. Statistical analysis

The parameters of the experiment are expressed as mean±SD. Statistical evaluation of the data was done using One-way ANOVA with the level of significance at $P<0.001$ using SigmaStat package version 3.1.

3. Results

3.1. Effect of C. amada leaf and rhizome extract on breast cancer cells

The result of MTT, SRB and LDH release assays revealed that the methanol extract of the leaves and the rhizomes of *C. amada* Roxb decreased the percent viability of all the cells but to different extent. The extract was found to induce more cytotoxicity towards cancer cell lines MCF-7 and MDA MB 231. The extract showed less cytotoxicity towards non-cancerous breast cell line HBL-100. The effect induced by the extracts is also similar to that of the standard chemotherapeutic drugs tamoxifen and etoposide which are commonly used in the treatment of estrogen receptor positive and triple negative breast cancer. The combination of the extracts along with the standard drugs also induced more cytotoxicity in cancerous cells.

These results revealed that the extracts induced cell death in the breast cancer cell lines. In order to find the mechanism of cell death induced by the extracts, various staining techniques were carried out to observe various morphological and nuclear changes associated with apoptosis (Figure 1).

Various morphological and nuclear changes characteristics of apoptosis such as membrane blebbing, chromatin condensation, nuclear fragmentation were observed by various staining techniques. The results

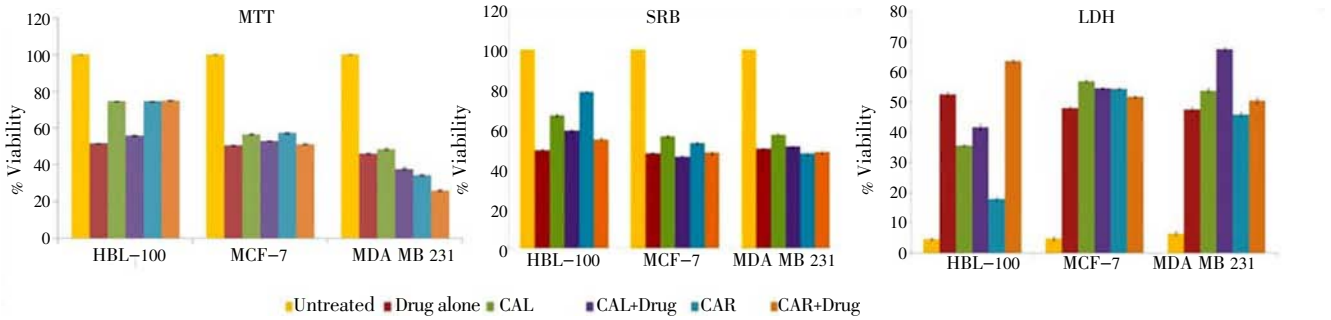

Figure 1. Effect of *C. amada* leaf and rhizome extract on cell survival.

CAL: methanol extract of *C. amada* leaves; CAR: methanol extract of *C. amada* rhizomes.

Table 1

Apoptotic cells/100 cells under observation of morphological and nuclear changes associated with apoptosis.

Treatment groups		HBL-100		MCF-7		MDA MB 231	
		Control	Tamoxifen treated	Control	Tamoxifen treated	Control	Etoposide treated
AO/EtBr staining	Cells	8±1	53±1[a]	13±1	48±2[a]	8±1	45±2[a]
	Cells+leaf extract	28±1[a,d]	37±1[a,b,c]	43±1[a]	58±2[a,b,c]	57±1[a]	64±3[a,b,c]
	Cells+rhizome extract	23±1[a]	39±1[a,b,c]	44±1[a]	57±1[a,b,c]	55±1[a]	62±1[a,b,c]
Giemsa staining	Cells	9±1	49±2[a]	13±1	51±2[a]	7±1	44±2[a]
	Cells+leaf extract	25±4[a]	42±1[a,b,c,d]	47±1[a]	56±1[a,b,c]	59±2[a]	64±1[a,b,c]
	Cells+rhizome extract	25±2[a]	38±1[a,b,c]	49±1[a]	57±1[a,b,c]	56±2[a]	59±3[a,b,c]
EtBr staining	Cells	9±1	53±1[a]	13±1	50±3[a]	8±2	46±1[a]
	Cells+leaf extract	27±4[a]	40±3[a,b,c]	45±1[a]	57±2[a,b,c]	62±1[a,d]	65±2[a,b,c,d]
	Cells+rhizome extract	23±3[a]	38±2[a,b,c]	45±4[a]	55±1[a,b,c]	54±1[a]	58±2[a,b,c]
PI staining	Cells	5±2	52±1[a]	14±1	48±2[a]	7±1	45±3[a]
	Cells+leaf extract	26±1[a]	41±3[a,b,c,d]	44±4[a]	59±2[a,b,c]	61±4[a,d]	64±2[a,b,c,d]
	Cells+rhizome extract	22±3[a]	36±1[a,b,c]	43±3[a]	57±2[a,b,c]	53±1[a]	57±1[a,b,c]
Hoechst 33342 staining	Cells	8±1	49±1[a]	14±1	50±1[a]	7±2	44±1[a]
	Cells+leaf extract	27±3[a]	45±2[a,c,d]	48±1[a]	54±2[a,b,c]	62±3[a,d]	65±1[a,b,d]
	Cells+rhizome extract	25±1[a]	39±1[a,b,c]	47±1[a]	57±4[a,b,c]	55±1[a]	57±4[a,b]

[a]: Statistically significant ($P<0.05$) compared to untreated control; [b]: Statistically significant ($P<0.05$) compared to drug alone treated group; [c]: Statistically significant ($P<0.05$) compared to the respective extract treated group; [d]: Statistically significant ($P<0.05$) compared to the rhizome extract treated group.

showed that the administration of methanol extract of leaves and the rhizomes of *C. amada* induced apoptosis in both the breast cancer cell lines. The results also confirmed the differential effect induced by the extracts in cancerous and non-cancerous cells (Table 1).

4. Discussion

The results of our experiments also correlated with the below findings by other researchers. The methanol extract of seed oil of *Pongamia glabra* showed antiproliferative effect on breast cancer cell line MCF-7 and cervical cancer cell line Hela with IC_{50} of 6 mg/mL after 48 h treatment[15]. The sub-fractions SC/D-F8, F9 and F10 of dichloromethane extract of *Strobilanthes crispus* exhibited cytotoxicity towards breast cancer cell lines MCF-7 and MDA MB 231 and prostate cancer cell lines PC-3 and DFU-145. F13, F14, F15 induced maximum cell death in MCF-7 whereas F1 was more cytotoxic towards MDA MB 231 cells and non-cytotoxic towards MCF-7[16].

The crude methanol extract of stem and leaves of *Kedrostis foetidissium* exhibited growth inhibitory activity on the breast cancer cell line MCF-7 and YMB-1 cell lines[17]. The ethyl acetate extract of *Chorisia* leaf treatment for 24 h to breast cancer cell line MCF-7 resulted in a dose and time dependent inhibition of the growth with IC_{50} of 5.2 μg/mL[18]. Furanodiene, an active component from *Curcuma wenyujin* increased the LDH release in both breast cancer cell lines MCF-7 and MDA MB 231 in a dose dependent manner[19]. The administration of magnetic nanoparticles to the MCF-7 breast cancer cells for 24 to 72 h increased the percent LDH release[20].

The methanolic extract of leaves and fruits of *Ligustrum vulgare* L. induced apoptosis in human colon carcinoma cells HCT-116 as evident by observation of apoptotic nuclear changes by AO/EtBr staining[21]. Apoptotic bodies and nuclear condensation were observed by Giemsa staining in MOLT-4 leukemic cell line after exposure to Terpinen-4-ol, a terpene found in the rhizome of *Zingiber montanum*[22]. Various nuclear changes associated with apoptosis such as blebbing, chromatin condensation and nuclear shrinkage

was observed by Hoechst 33258/PI staining in ethanol induced neuronal PC-12 cell line[23].

Our results also showed that the extracts induced cell death in breast cancer cells which is independent of estrogen receptor status of the cells and the mechanism of cell death induced by the extracts is mediated through apoptotic pathway.

C. amada was found to possess anticancer properties and it could be used as potent drug candidate to be used in pharmaceutical preparations. It is recommended that the extracts can be used as a supportive therapy for the treatment of cancer which selectively kills the cancer cells and rendering protection to normal cells.

Conflict of interest statement

We declare that we have no conflict of interest.

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